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CONTENTS

WILLIAM M. GIBBS, H. W. BATCHELOR, AND T. N. SICKELS. Surface Tension and Bacterial Growth 393

IVAN C. HALL. The Reduction of Selenium Compounds by Sporulating Anaerobes 407

STEWART A. KOSER. Further Observations on Utilization of the Salts of Organic Acids by the Colon-Aerogenes Group. 409

JAMES M. SHERMAN. The Production of Catalase by an Anaerobic Organism 417

RALPH R. MELLON. Studies in Microbic Heredity. VII. Observations on the Genetic Origin of the Several Types of Fungi Found in the Lesions of Blastomycosis Hominis 419

INDEX TO VOLUME XI 435

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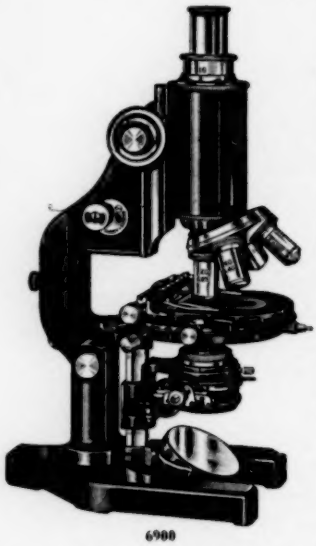

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SURFACE TENSION AND BACTERIAL GROWTH[1]

WILLIAM M. GIBBS, H. W. BATCHELOR, AND T. N. SICKELS

From the Department of Bacteriology, University of Idaho, and Moscow, Idaho, Agricultural Experiment Station

Received for publication May 15, 1925

HISTORICAL RÉSUMÉ

We have found only a few reports which bear directly on surface tension as a factor in bacterial growth.

Larson, Cantwell, and Hartzell (1919), studied the effect of lowered surface tension on the growth and characteristics of certain organisms, and concluded that the growth of bacteria in ordinary broth was greatly influenced by the surface tension of the medium. The surface tension was lowered by additions of castor oil soap and measured by the drop weight method. The standard broth which was employed had a surface tension of 59 dynes per square centimeter. *B. subtilis* did not form a pellicle when the surface tension was reduced to 45 dynes, but grew down in the body of the medium. They concluded that all pellicle formers ceased to grow at the surface when the surface tension was below 45 dynes per centimeter. *B. subtilis* was found to form spores more slowly in a medium of lowered surface tension.

Some anaerobes, particularly *B. tetani*, were found to grow aerobically in a medium of lowered surface tension. The authors suggest that the favorable action of the oil seal in anaerobiosis is probably due to the lowered surface tension. They are inclined to believe that the toxicity of the depressant is a factor of more or less importance. In a later report Larson (1921) found the surface tension of ordinary broth to be about

[1] Published with the approval of the Director of the Idaho Agricultural Experiment Station as scientific paper No. 38, College of Agriculture and Agricultural Experiment Station, Moscow, Idaho.

59 dynes, and of inorganic media with glycerol as a source of carbon, 73 dynes. The surface tension was depressed by additions of castor oil soap, giving a range from 32 to 73 dynes per square centimeter. The growth of pneumococcus and streptococcus was depressed at a surface tension of 50 dynes and lower. The limit of *B. anthracis* was sharply defined at 46 dynes. The intestinal bacteria grew well at lowered surface tension. The ability of this group to develop in media of low surface tension was thought to be related to the resistance of the group to bile, which has a low surface tension. The concentration of the colon-typhoid group of bacteria at the surface of the medium was attributed to the concentration of the surface tension depressants at the surface. Pellicle formers were found to grow throughout the body of the medium when the surface tension was reduced, while non-pellicle formers were found to develop pellicles after long cultivation in media of high surface tension. Symbiosis and antibiosis, were, in some cases, attributed to surface tension.

Ayers, Rupp, and Johnson (1923) studied the effect of surface tension on the streptococci. Castor oil soap, sodium glycocholate, iso-amyl alcohol, and sodium oleate were used as depressants. The basic medium employed had a surface tension of 59.6 dynes, and was depressed as low as 40 dynes. These authors conclude that some species of streptococci are retarded in growth when the surface tension of the medium is lowered to 53 dynes, and suppressed at 45 dynes, while others are retarded at 43 dynes. Still other species are depressed at 40 to 41 dynes. In general 40 dynes prevented growth. *Streptococcus pyogenes* was the most susceptible to lowered surface tension, and *Strept. lactis* least affected. Some toxicity of the depressants was noted.

Mellon, Hastings, and Anastasia (1924) studied the cohesive power responsible for the spontaneous agglutinability of certain bacteria. They found that this power could be diminished or entirely eliminated by varying the surface tension of the solution. Strains which immediately agglutinated, or failed to produce emulsions, were found to emulsify readily in a solution contain-

ing sodium oleate. This was attributed to the interfacial tension between organism and solution.

EXPERIMENTAL METHODS

Preparation of broth. The stock broth used in the experiments was prepared as follows: Parke Davis and Company, pepton 10 grams, beef extract 3 grams, distilled water 1000 grams. These ingredients were cooked together in the steamer one and one-half hours, the reaction adjusted to pH 7.0 and steamed for another half hour, then cooled and filtered under pressure through macerated filter paper. Broth prepared in this manner had a surface tension of 48 to 50 dynes per square centimeter.

Surface tension depressants. Soaps were prepared from castor oil, cocoanut oil, olive oil, and palmitic acid. The oils were saponified by adding an alcoholic solution of KOH in excess and heating under a reflux condenser for several hours, then allowing to stand overnight. The solution was neutralized to phenolphthalein by a solution of HCL in absolute alcohol and filtered hot through filter paper to remove chlorides. It was then concentrated to small volume and poured into an excess of saturated solution of sodium chloride in water. The precipitated glycerides were collected on filters and washed with saturated sodium chloride solution, then dried at 28°C. and finally dried at 60°C. They were then dissolved in absolute alcohol, filtered, again evaporated and dried, and again taken up in absolute alcohol and filtered. They were then dried at 28°C. and finally at 60°C. and stored for use.

Stock solutions of the four soaps were prepared by dissolving 2 grams in 100 cc. of distilled water. These 2 per cent solutions were then cooled to just above the freezing point and filtered under pressure through macerated filter paper. This cooling and filtering process was repeated until the solutions were perfectly clear and gave little or no precipitate when added to the stock broth. These 2 per cent solutions were used as bases and were added to broth or other media by pipette. A broth referred to as containing 5 per cent soap contains 5 cc. of this 2 per cent soap solution per 100 cc. of medium.

The four soaps mentioned were used throughout the work, in order that any phenomena observed, in reality due to the nature of the depressant, would not be ascribed to surface tension. This precaution was justified in nearly every experiment.

The surface tension depressing efficiency of these soaps varied considerably, not only in the actual depression possible, but in the proportion of soap necessary to produce it. With each soap there was a maximum concentration, beyond which further additions had little effect on the surface tension. With castor soap this was 1 per cent, with olive soap about 2 per cent, with palmitic soap 6 per cent, and with cocoanut soap as high as 10 per cent. From the basis of the amount of soap necessary to produce a given depression, cocoanut soap was the most efficient, 0.1 per cent giving a depression of 7 to 8 dynes and 1 per cent giving a depression of 18 dynes. Olive soap was only slightly less efficient. Palmitic soap was used in concentrations as high as 5 per cent without securing any more depression of the surface tension than with olive or castor soap at 1 per cent. Cocoanut produced the lowest surface tension of any of the soaps but was less satisfactory, due to the fact that it sometimes produced turbidity or precipitates. Castor and palmitic soaps gave perfectly clear solutions in all concentrations used and olive soap slightly opalescent solutions but without precipitates.

The possible toxicity of depressants is an important factor. These four soaps varied considerably in this respect to different organisms. Some organisms were very susceptible to a given soap, others apparently not at all.

Surface tension measurement. The surface tension was determined by the film method as outlined by Fahrenwald (1922). In one experiment, the drop weight method was also employed for the sake of comparison. The film method has the advantage that several determinations can be made in a relatively short time, not at the expense of accuracy. It is also possible by this method to distribute the surface tension depressants, which accumulate at the surface, throughout the body of the medium, thus enabling one to check on separate determinations. It will appear that the surface tension varies slightly with each measure-

ment of the same solution, and an equilibrium cannot be obtained. This may be avoided by gently stirring the depressants into the body of the medium, then quickly measuring the tension. A second measurement will then be found to check the first. Results obtained by the method of Fahrenwald are lower than those obtained by the drop weight method.

EXPERIMENTAL DATA

Escherichia coli and surface tension

Beef extract broth containing the various depressants was inoculated with a twenty-four-hour culture of *Escherichia coli* and incubated at 28°C. The flasks were so arranged that the surface tension could be checked at intervals to determine the effect of the metabolism of the organism on the surface tension of the medium. The pH of the broth was 7.0. Table 1 shows the arrangement of the cultures, the surface tension, and the hydrogen ion concentration. Each soap had a series not inoculated in order to trace changes due to factors other than bacterial development. The surface tension was determined by the method of Fahrenwald and by the drop weight method. The results are shown in table 1 and chart 1.

It is seen from the data in the table and chart that the depressants gave a range in surface tension, as determined by the film method from 41.2 to 51.2 dynes per cubic centimeter. The same broths as determined by the drop weight method gave a range of 54.2 to 63.2 dynes per cubic centimeter. The latter method in this case gives, in general, results about 12 dynes higher than the film method. It will be noted that the difference in the two methods remains somewhat constant throughout. In discussing the results the data obtained by the film method will be taken.

The surface tension in the uninoculated control broth increased 1.5 dynes per cubic centimeter during the ten-day period. The hydrogen ion concentration remained constant. The uninoculated broths which received depressants gave a general small increase in surface tension during the period, an average increase

TABLE 1

Escherichia coli and surface tension

TREATMENT 2 PER CENT SOLUTION PER 100 CC. BROTH	TIME OF INCUBATION	pH	SURFACE TENSION PER CENTIMETER			
			Inoculated.		Not inoculated	
			Film method	Drop weight method	Film method	Drop weight method
	days		*dynes*	*dynes*	*dynes*	*dynes*
None	0	7.0	51.2	63.2	51.2	63.2
	1	7.1	54.3	64.4	53.4	63.9
	2	7.8	52.5	61.9	51.4	61.4
	3	8.0	51.8	62.2	52.4	63.7
	5	8.3	51.9	65.6	52.3	63.5
	7	8.3	52.6	65.6	52.0	64.8
	10	8.3	54.8	70.0	52.7	64.3
Castor oil soap, 0.1 cc	0	7.0	44.9	58.3	44.9	58.3
	1	7.3	48.5	60.8	47.8	58.3
	2	7.7	49.4	59.3	46.6	56.6
	3	8.1	50.0	61.5	47.3	58.7
	5	8.2	51.4	64.1	47.1	59.7
	7	8.4	52.8	65.6	47.2	57.7
	10	8.4	53.4	69.1	47.5	63.5
Olive oil soap, 0.2 cc	0	7.0	46.8	59.6	46.8	59.6
	1	7.0	49.7	62.9	49.1	62.2
	2	7.6	50.3	60.7	49.7	61.4
	3	8.1	49.6	61.5	49.0	60.8
	5	8.2	50.4	64.1	48.7	62.5
	7	8.3	51.4	64.6	49.1	60.3
	10	8.3	51.0	68.2	48.5	63.5
Cocoanut oil soap, 1 cc	0	7.0	41.2	54.2	41.2	54.2
	1	7.2	45.1	56.5	44.3	55.2
	2	7.6	49.1	61.4	43.5	53.8
	3	8.1	49.6	60.8	44.4	54.5
	5	8.3	51.8	64.1	43.2	54.8
	7	8.4	51.6	64.0	44.9	53.6
	10	8.4	52.0	69.1	43.8	58.7
Palmitic soap, 3 cc	0	7.0	43.6	57.7	43.6	57.7
	1	7.1	47.1	60.1	48.3	62.2
	2	7.8	48.1	61.4	47.2	61.4
	3	7.9	47.5	59.4	46.1	57.5
	5	8.1	49.2	62.5	46.1	56.5
	7	8.2	50.1	63.2	46.4	59.0
	10	8.3	53.0	63.5	46.8	61.4

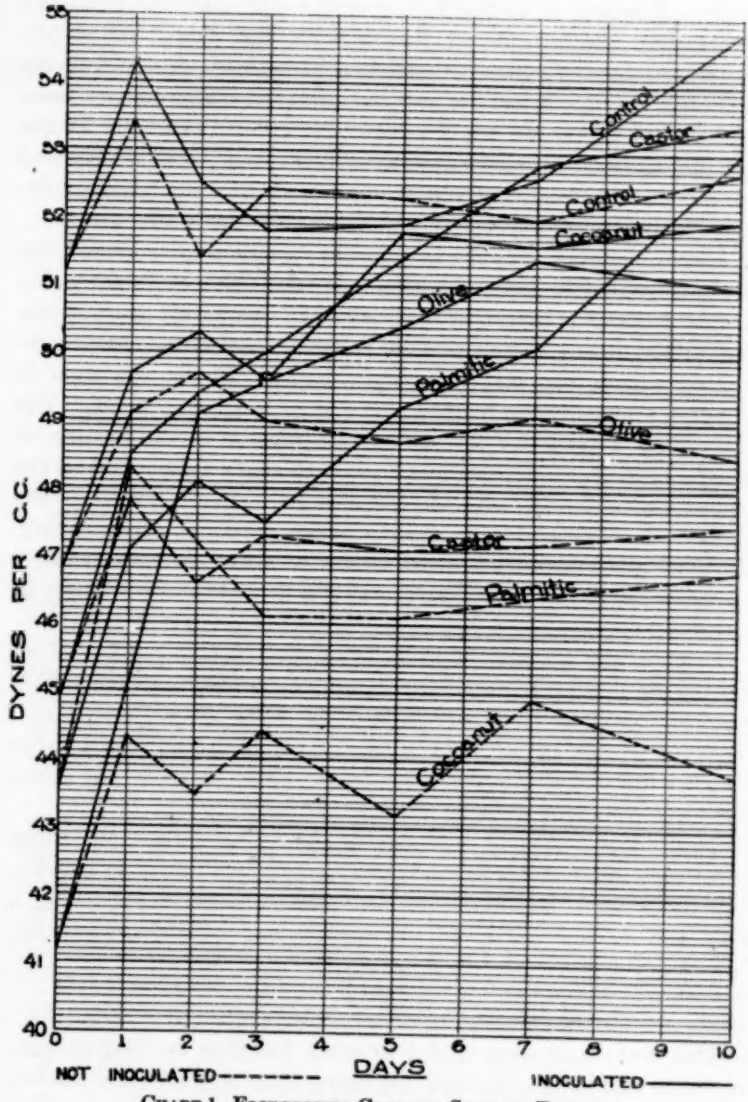

CHART 1. ESCHERICHIA COLI AND SURFACE TENSION

of 2 to 3 dynes, while the hydrogen ion concentration remained constant. The inoculated flasks, with the exception of the untreated control, gave an increase in surface tension varying from 4.2 to 10.8 dynes per cubic centimeter. The control inoculated flask increased but 3.6 dynes per cubic centimeter in the ten days. The maximum increase in surface tension occurred in the cocoanut treated series which also had the greatest depression at the start.

It will be noted that the increase in surface tension during the ten-day period is inversely proportional to the surface tension at the beginning of the experiment. In nearly all cases in the inoculated series the maximum increase in surface tension occurred within the first forty-eight hours. There was then in general a steady climb until the end of the experiment. The data show a tendency for the surface tension in the inoculated broths containing depressants to approximate that found in the uninoculated broth with no depressant, within seven to ten days. This shows that growth characteristics after several days' incubation cannot be attributed to a surface tension found at the onset of the experiment. This is brought out strikingly in chart 1.

Even in the absence of inoculation the surface tension of all broths containing depressants showed a decided increase within twenty-four hours. There was then some slight fluctuation throughout the period, but in no case did the surface tension drop to the level found at the onset, or increase to the point found in the control broth without depressant.

There was a steady increase in pH in all inoculated tubes until they reached 8.3 to 8.4 at the tenth day. Thus we find the surface tension and hydrogen ion concentration approaching a constant as the result of the metabolism of *Escherichia coli* regardless of the initial surface tension.

Growth of aerobes in broth of varying surface tension

The four soaps were added to stock broth in varying concentrations and hydrogen ion concentration and surface tension determined. Each experiment was so arranged that the pH of

all the tubes was approximately the same at the start. Tubes of each concentration were then inoculated with a twenty-four-hour culture of each of the following organisms: *B. subtilis*, *B. anthracis*, *Serratia marcesens*, *Pseudomonas aeruginosa*, *Eberthella typhi*, and *Staphylococcus aureus*. The tubes were then incubated at 28°C. and observations made after twenty-four and forty-eight hours and again at the end of one week.

The surface tension and soap additions are shown in table 2.

All the soaps except palmitic seemed to be somewhat toxic to both *B. subtilis* and *B. anthracis*. Palmitic proved to be stimulating to *B. subtilis* in forty-eight-hour incubation, and the organism produced a heavier pellicle with this soap at 33 dynes

TABLE 2

Surface tension of broth containing depressants

	CASTOR SOAP			OLIVE SOAP			PALMITIC SOAP			COCOANUT SOAP		
Cubic centimeter of 2 per cent solution per 100 cc. broth.....	0.1	0.5	1.0	1.0	3.0	5.0	1.0	3.0	5.0	1.0	3.0	5.0
Surface tension, dynes per square centimeter.	43	35	33	33	32	31	37	34	33	44	38	30

Surface tension of control broth, 49 dynes per square centimeter.

than in the control of 49 dynes. At the end of a week this difference was not noted, the growth being about the same as that in the control. *B. anthracis* failed to develop well in any of the tubes containing soap.

Results were somewhat striking with *Pseudomonas aeruginosa* and *Serratia marcesens*, particularly the former. Both organisms showed stimulated pigment formation, heavier pellicles, and less growth in the body of the media, in all tubes which contained soap. Since castor oil soap was not toxic to *Pseudomonas aeruginosa* it was later employed in concentrations of 1, 3, and 5 per cent. These higher concentrations of the soap did not produce any further depression of the surface tension, yet the stimulation of pigment formation and pellicle, and diminution of growth throughout the body of the medium was more marked than in the previous concentrations. The observed stimulation

was then due to the nature of the depressant rather than to the lowered surface tension. This shows the importance of using several depressants in order not to ascribe observed phenomena to the surface tension rather than the nature of the depressant.

Eberthella typhi was apparently unaffected by any of the soaps, growth in the treated tubes differing in no way from the control. The soaps were toxic to *Staphylococcus aureus* in the higher concentrations, and no differences could be ascribed to surface tension.

These results do not agree with those of Larson who states that all pellicle formers cease to grow at the surface when the surface tension is below 45 dynes. The above experiments were repeated many times and in every case, especially with the two pigment formers, the pellicle formation was stimulated in the presence of the soap. This stimulation is not ascribed to surface tension. It must be borne in mind that the lowest surface tension obtained in the above experiment, 31 dynes per square centimeter, would be about 42 to 43 dynes as determined by the drop weight method.

Growth in Uschinsky's medium. The same organisms used in the above experiment were used in a similar experiment employing Uschinsky's medium instead of the stock broth. The surface tension varied from 26 to 71.6 dynes per square centimeter. Control flasks showed that the surface tension of the medium remained approximately the same after sterilization as before.

Pseudomonas aeurginosa and *Serratia marcesens* developed very intense pigments and grew well. The latter organism produced very little pigment in the medium containing cocoanut soap, but the pigment was intense in the other tubes. The differences noted could in no way be ascribed to the lowered surface tension.

Growth of anaerobes

Soaps were added to the stock broth containing 1 per cent glucose. The surface tension is shown in table 3.

The surface tension of the control broth was 53 dynes. Tubes of each concentration of each soap were then inoculated with the following anaerobes: *C. histolyticum*, *C. welchii*, *C. sporogenes*, *C. bifermentans*, *B. bellonensis*, *C. oedematis-maligni*, *C. oedematiens*, and two strains of *C. botulinum*. Controls consisted of an aerobic control, an anaerobic control, and a toxicity control for each organism. The aerobic control consisted of a tube of the glucose broth inoculated with the organism in the usual manner. The anaerobic control consisted of a tube of the glucose broth boiled for ten minutes, plunged in cold water, inoculated, and then sealed with a layer of sterile paraffin oil. The toxicity control consisted of tubes of the glucose broth containing the various soaps, boiled, and after cooling and inoculating, sealed with paraffin oil.

TABLE 3

Surface tenstion of glucose broth containing depressants

	CASTOR SOAP			OLIVE SOAP			PALMITIC SOAP		
Cubic centimeter of 2 per cent solution per 100 cc. broth	0.1	0.4	0.8	0.1	0.4	0.8	1.0	3.0	5.0
Surface tension, dynes per cubic centimeter	52	43	39	51	42	38	48	44	40

In no case did any of the anaerobes show any development in the tubes not sealed with paraffin oil. All showed excellent growth in the tubes treated with the paraffin seal. The toxicity controls in general gave good growth, some showing less growth than the controls. *C. botulinum* was completely inhibited by both palmitic and castor soaps, but grew well in the olive soap. This would indicate that lowered surface tension within the limits of this experiment, does not bring about the development of anaerobes under aerobic conditions.

Surface tension and nitrogen fixation by Azotobacter chroococcum

Ashby's medium received various concentrations of soap, as indicated in table 4, and was then inoculated with a soil sus-

TABLE 4

Surface tension and nitrogen fixation by Azotobacter chroococcum

NUMBER	TREATMENT	SURFACE TENSION PER CUBIC CENTIMETER	NITROGEN FIXED PER 100 CC.	
				Average
	per cent	*dynes*	*mgm.*	*mgm.*
1–2	Castor 0.1	54.5	9.1 9.3	9.2
3–4	Castor 0.5	43.0	8.3 9.1	8.67
5–6	Castor 1.0	40.3	11.8 10.4	11.1
7–8	Cocoanut 1.0	54.5	10.3 11.5	10.9
9–10	Cocoanut 3.0	40.3	10.1 12.0	11.05
11–12	Cocoanut 5.0	34.8	10.6 9.7	10.15
13–14	Palmitic 1.0	48.0	10.2 10.1	10.15
15–16	Palmitic 3.0	45.8	10.7 10.9	10.8
17–18	Palmitic 5.0	38.2	11.2 11.6	11.4
19–20	Olive 0.1	48.0	10.4 9.7	10.05
21–22	Olive 0.5	39.8	9.4 10.2	9.8
23–24	Olive 1.0	38.2	11.7 9.4	10.55
25–26	Controls	74.2	10.8 11.7	11.25

pension containing Azotobacter. The flasks were then incubated at 25°C. for two weeks and the total nitrogen determined.

The Azotobacter film was uniform, pigmented, and heavy on all flasks, little or no difference being noted. Table 4 shows the surface tension of the media and the amount of nitrogen fixed.

It is obvious that neither the lowered surface tension nor the presence of the depressants have materially affected nitrogen fixation by *Azotobacter chroococcum*.

Oil seals

Interesting results were obtained with various oil seals sometimes employed in the cultivation of anaerobes in broth. The surface tension of ordinary paraffin oil was found to be about 28 dynes, olive oil 34 dynes, and castor oil 35 dynes. By using these oils as seals over ordinary broth the difference in tension of the phases is lessened and consequently the tension is lowered. No change was noted in the development of an ordinary pellicle forming organism under such a seal, but *Eb. typhi* formed a well-developed pellicle at the two phases, particularly in the case of olive oil. This did not develop in the case of paraffin oil. Azotobacter developed a splendid film under a half-inch layer of paraffin oil, and fixed as much N. as in the control flasks. Other data with reference to interfacial tension and bacterial growth will be presented at a later date.

SUMMARY

The foregoing experiments show that one of the most important factors in work on the relation of surface tension to bacteriological activities is the nature of the depressants employed. Soaps were found to be very satisfactory depressants because a very small amount produced a material depression in the surface tension. There is a considerable difference in various soaps, not only in their ability to depress surface tension, but in the effect on the organisms. Error in observations can only be avoided by using several soaps in concentrations such that each produces similar depression in the surface tension. If an observed phenomenon does not occur with several depressants, at approximately the same surface tension, it cannot be cor-

rectly ascribed to surface tension, but must be due to the nature of the depressant. Keeping this in mind, and using four depressants, no case has been found in the preceding experiments in which variation from control might better be attributed to surface tension than to the influence of the depressants themselves.

No observation has been made which would lead to the conclusion that pellicle formation is suppressed at lowered surface tension. Organisms produced pellicles at a surface tension as low as 32 degrees per square centimeter as determined by the method of Fahrenwald. This would correspond to a surface tension of 42 to 44 dynes as determined by the drop weight method.

Anaerobic bacteria failed to develop under aerobic conditions even when the surface tension was greatly lowered.

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THE REDUCTION OF SELENIUM COMPOUNDS BY SPORULATING ANAEROBES

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One of the first duties of any author who refers to a living organism by its generic and specific names is to make sure by all means at his command of the identity of the form in question. This principle evidently was not followed by Levine (1925) in the case of a recent paper, containing a list of sixteen sporulating obligate anaerobes which he tested for their reduction of sodium selenite. These cultures were obtained from the collection formerly in the American Museum of Natural History in New York City, now maintained in Chicago, and were apparently accepted at their face value without any effort to check up their identity by criteria which are now well established. There is no practice so conducive to confusion or so detrimental to progress in the science of bacteriology as the willingness of workers to accept uncritically the names upon so called "authentic" stock cultures.

It happens that the writer (1922) studied ten of the cultures in question, not for their action upon selenium, but for their purity and identity. Only one of the ten, i.e., *B. sporogenes* 120, was pure and correctly named! "*B. welchii* 500" contained *B. welchii* mixed with *B. sporogenes;* "*B. welchii* 521" probably contained *B. welchii* but only *B. sporogenes* was isolated. "*B. tetani* 1" contained no pathogenic organisms but consisted of a highly putrefactive species that I have designated *B. tyrosinogenes* because of its marked liberation of tyrosin from protein media. This is the only strain of this species that has ever been recognized. "*B. tetani* 274" contained no tetanus bacillus but consisted of an unidentified anaerobe and *B. sporogenes.* The

remaining five cultures, though evidently pure, consisted of *B. sporogenes*.

The correspondence between the museum's designations and those of the writer, as published in 1922, is as follows:

Museum's designations	*Writer's designations*
B. welchii 521	*B. welchii* 2 contaminated with *B. B. sporogenes* 72
B. welchii 500	*B. welchii* present (?) contaminated with *B. sporogenes* 42.
B. welchii 20	*B. sporogenes* 114
B. feseri 48	*B. sporogenes* 122
B. tetani 274[1]	*B. sporogenes* 88, also unidentified anaerobe, not *B. tetani*
B. tetani 1	*B. tyrosinogenes* 106
B. sporogenes 120	*B. sporogenes* 84
B. oedematis 421	*B. sporogenes* 121
B. oedematis maligni 485	*B. sporogenes* 90
B. botulinus 595	*B. sporogenes* 44, 46 (?)

The actual serologic identity of the sporogenes strains was later shown by Hall and Stark (1923) by means of the agglutination test. The fact that all of the anaerobes tested by Levine reduced sodium selenite at 1:50,000 suffers considerable limitation when interpreted in the light of these facts. We may readily agree that *B. sporogenes* reduces sodium selenite but any conclusions regarding the other species of anaerobes mentioned must await more critical experiments.

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[1] This culture came to the writer indirectly from the museum through the Department of Bacteriology and Hygiene at the University of Chicago.

FURTHER OBSERVATIONS ON UTILIZATION OF THE SALTS OF ORGANIC ACIDS BY THE COLON-AEROGENES GROUP

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A previous study of the utilization of salts of organic acids by the colon-aerogenes group has shown that *Bacterium aerogenes* and its allies are capable of breaking down the sodium, potassium and ammonium salts of citric acid while *Bacterium coli* from the intestines of man and higher animals does not possess this property. Of the eighteen different organic acids employed, citric acid was the only one which permitted a clear cut distinction between these two types.

The present investigation represents a continuation of the previous work and in this instance attention has been centered upon several organic acids whose structure somewhat resembles that of citric acid. Information concerning the behaviour of the colon group toward these acids appeared desirable from several viewpoints. In the first place an opportunity was offered for studying the relationship of chemical constitution to bacterial utilization, and secondly, there was the possibility that structurally related acids might afford the same separation between the coli and aerogenes types and thus be of value as an additional means of separating the main sections of the group. In addition to citric acid, aconitic, tricarballylic, glutaric and adipic acids were employed. These were obtained from either American or British chemical houses or from the Chemistry Department of the University of Illinois.

In a recent study of the organic acids for differentiating various bacterial types, Brown, Duncan, and Henry (1924)

mention the employment of the sodium salts of aconitic, glutaric and citric acids in addition to many others. The organisms used in their study were chiefly members of the paratyphoid group though several colon-aerogenes cultures and others were included. They confirm the writer's observations on the value of sodium citrate for differentiating the colon group. They further state that the salts of aconitic and glutaric acids are apparently not readily decomposed by any member of the Salmonella group or allied types, though they do not mention the colon group specifically in this connection.

It should be noted that their method of testing utilization of the acids differed from that previously employed by the writer (Koser, 1923–1924). Instead of using a synthetic medium and supplying the acids as the sole source of energy, they added them to either nutrient broth or a pepton solution. Under these conditions it was found that gas production, a change in reaction, or a more luxuriant or retarded growth were not sufficiently reliable criteria of utilization. They then resorted to precipitation by means of lead acetate. This procedure afforded a reliable indication of utilization of many of the acids and a number of very interesting distinctions were brought out, chiefly between members of the paratyphoid group.

In the present study, the procedure for testing utilization of the acids was similar to that employed in former work (Koser, 1923). The basic medium consisted of 0.5 per cent sodium chloride, 0.02 per cent magnesium sulphate, 0.1 per cent dibasic potassium phosphate and 0.1 per cent ammonium acid phosphate and to this the various organic acids were added, one at a time, in amounts sufficient to give a concentration of 0.2 per cent. Each solution was then brought to pH 6.8 by the addition of sodium hydroxide solution so that the resulting media contained somewhat over 0.2 per cent of the sodium salt of each acid. Media were tubed in 5 to 6 cc. amounts and sterilized in the autoclave at 15 pounds extra pressure for fifteen to twenty minutes.

Representative cultures of each section of the colon-aerogenes group were tested in the foregoing media. The coli

section was represented by 40 cultures recently isolated from fecal material, the aerogenes section by 35 cultures obtained from soil. The soil strains had been isolated several years previously and kept in stock on agar slants for two to three years. Most of them failed to liquefy gelatin and thus would be classed as of the aerogenes rather than the cloacae type. Also, a number of other cultures of soil origin were used. These resembled the typical intestinal *Bacterium coli* in respect to the methyl red and Voges-Proskauer tests, but had been obtained from a different natural habitat and could easily be distinguished on the basis of the citrate test. In the following table they are designated the "intermediate type."

After inoculation of the different organic acid media, records of growth as determined by visible turbidity were made daily for the first four or five days and thereafter at more infrequent intervals up to thirty days. Determinations of the hydrogen ion concentration by the colorimetric method with the usual indicators were also frequently made as an aid in measuring utilization of the acids. The temperature of incubation was 30°C. throughout the work.

The results summarized in Table 1 represent development at the fourth day after inoculation. With certain exceptions which will be mentioned later, further incubation failed to bring out any striking changes from those observed at this time so that the record of results at the fourth day gives a fairly accurate picture of the development of the organisms in the different organic acid solutions. Citric acid was used for purposes of comparison in addition to the controls given at the foot of the table. The results secured with citric acid, employed as its sodium salt, are similar to those previously reported.

As shown in the first column of the table, *Bact. coli* was unable to make use of any of the acids with the possible exception of glutaric, though here the results are not conclusive since development was slight. Some of the aerogenes cultures and the intermediate type utilized tricarballylic and aconitic acids, though this ability was not possessed by all of them. Glutaric acid was utilized sparingly if at all, while adipic acid gave nega-

TABLE 1

Utilization of certain organic acids by the colon-aerogenes group

ORGANIC ACIDS EMPLOYED AS THE SODIUM SALT	BACT. COLI; 40 STRAINS; METHYL RED + VOGES-PROSKAUER 0; HUMAN INTESTINE	INTERMEDIATE TYPE; 21 STRAINS; METHYL RED + VOGES-PROSKAUER 0; SOIL	AEROGENES SECTION; 35 STRAINS; METHYL RED 0, VOGES-PROSKAUER +; SOIL
Citric	(40) negative	(21) +++	(35) +++
Tricarballylic	(40) negative	(12) negative (9) +++	(29) negative (2) ? or + (4) +++
Aconitic, I	(38) negative (2) +	(18) ? or + (3) +++	(2) negative (22) ? or + (11) +++
Aconitic, II	(40) negative	(12) negative (6) ? or + (3) +++	(8) negative (16) ? or + (11) +++
Glutaric, I	(6) negative (34) ? or +	(6) negative (15) ? or +	(15) negative (20) ? or +
Glutaric, II	(12) negative (28) ? or +	(11) negative (10) ? or +	(26) negative (9) ? or +
Adipic	(40) negative	(21) negative	(31) negative (4) ?
Controls:			
1. Basic medium + 0.2 per cent glucose	(40) +++	(21) +++	(35) +++
2. Basic medium only, no added source of energy	(40) negative	(21) negative	(34) negative (1)* ? or +

Increasing luxuriance of growth from slight to heavy turbidity is indicated by the plus signs, +, ++, and +++. No evidence of growth is designated "negative." The numerals in brackets preceding these signs show the number of cultures in each case.

The observations given here are readings taken on the fourth day after inoculation. Incubation at 30°C.

* This one culture gave questionable or slight growth in the basic medium; upon several different tests, however, it failed to show growth upon successive cultures in this solution.

tive results. All of these acids failed to yield the clear cut distinction shown by citric acid.

A comparison of the structural formulae of the several acids is of interest in connection with the question of their utilization. In the following list succinic acid is included for purposes of comparison though it was not used in the present work.

Citric	Tricarballylic	Aconitic
$CH_2 \cdot COOH$ | $C(OH) \cdot COOH$ | $CH_2 \cdot COOH$	$CH_2 \cdot COOH$ | $CH \cdot COOH$ | $CH_2 \cdot COOH$	$CH \cdot COOH$ ‖ $C \cdot COOH$ | $CH_2 \cdot COOH$

Succinic	Glutaric	Adipic
$CH_2 \cdot COOH$ | $CH_2 \cdot COOH$	$CH_2 \cdot COOH$ | CH_2 | $CH_2 \cdot COOH$	$CH_2 \cdot COOH$ | $(CH_2)_2$ | $CH_2 \cdot COOH$

It will be seen that the replacement of the hydroxyl group of citric acid by the hydrogen of tricarballylic renders the latter acid unavailable for many of the aerogenes and related cultures which readily attacked citric acid, while *Bacterium coli* refuses to grow in either case. In the dicarboxylic acid series represented by succinic, glutaric, and adipic acids, it was previously found (Koser, 1923) that succinic acid supports the growth of both *Bacterium coli* and *Bacterium aerogenes*. The higher members of this series seem to be less readily available, for glutaric acid permitted only a slight growth in some cases while adipic acid gave negative results. It is possible, also, that the results secured with glutaric acid may have been due to impurities and that this acid, like adipic, is not utilized.

The peculiar results given by glutaric acid and to some extent by aconitic acid deserve especial mention. Two samples of each of these acids were available for the tests and as somewhat different results were secured with each of the two different preparations, all are included in the table. In the first aconitic acid medium two strains of *Bacterium coli* developed

slightly, though all were negative in the medium prepared from the second sample. The intermediate type and the aerogenes cultures also gave somewhat different results in the two samples. In the first case only two strains of *Bacterium aerogenes* refused to grow, while 22 gave a questionable or slight growth and 11 produced a heavy vigorous growth with undoubted utilization of the acid. The questionable or slight growth was accompanied by little if any change in the hydrogen ion concentration, while the heavy growth always resulted in increased alkalinity. In the second sample eight of the aerogenes cultures were negative whereas six of these had shown questionable or slight growth before. The same eleven cultures which at first exhibited vigorous development again produced a heavy turbidity in the medium. A number of the "intermediate type" cultures have shown the same tendency toward questionable or slight growth in the presence of aconitic acid, though this was much more pronounced in the first sample.

Somewhat similar results were encountered with all of the cultures in the two samples of glutaric acid. In these cases the development was quite slow, a light turbidity appearing in from two to four days and in occasional cases not until the sixth or seventh day. A few aerogenes and several cultures of the intermediate group showed a somewhat heavier growth after two or three weeks, though this was accompanied by very little change in the hydrogen ion concentration.

In view of the foregoing results the purity of these two acids, especially the glutaric acid, may be questioned. The samples used were the best obtainable, but even though regarded as being chemically pure they may have contained traces of other compounds which affected the results in one way or another, either by supplying available foodstuffs and thus permitting some slight development or perhaps by retarding true utilization of the acid. There is some evidence to show that traces of impurities which can not be detected by the usual chemical or physical methods may be made evident by bacteriological procedures. Pfanstiehl and Black (1921) have pointed out the difficulty of determining the purity of sugars used in bacteriological work

since in many instances the chemical tests are insufficient. Kendall and Yoshida (1923) have shown that 0.0025 per cent of levulose in pepton solution may be detected readily by fermentation by *Bacterium coli* and that even the detection of 0.001 per cent was not impossible. Koser and Rettger (1919) found that the addition of 0.001 per cent of aspartic acid (neutralized) to a solution of inorganic salts and glycerol permitted slight development of a number of organisms. In a similar way Koser (1923) obtained growth by *Bacterium aerogenes* when 0.01 per cent sodium citrate was supplied as the source of energy, 0.001 per cent permitted some development though usually not enough for production of a visible turbidity.

The foregoing instances tend to show that the addition of very small quantities of available carbon- or nitrogen-containing compounds to a solution otherwise suitable for growth will be sufficient to bring about considerable multiplication. When submitting unknown compounds to bacteriological tests it follows that the determination of the melting point, to use but one example, may not be a sufficiently reliable criterion of purity and consequently results such as those secured with glutaric acid and in some cases with aconitic acid are open to several interpretations, since at present one can not be absolutely sure of the purity of the products tested.

It can be stated in a general way, however, that neither aconitic nor glutaric acids would be suitable for differentiation of the several sections of the colon group. Some strains of the aerogenes section fail to utilize aconitic acid and thus can not be distinguished from *Bacterium coli* by this means, while both the coli and aerogenes types exhibit about the same deportment toward glutaric acid.

SUMMARY

The utilization of several organic acids of the dicarboxylic and tricarboxylic series by *Bacterium coli*, *Bacterium aerogenes*, and related types has been studied.

Although several of these acids are structurally quite similar to citric acid, none of them afforded the same distinction be-

tween the intestinal *Bacterium coli* and other members of the colon-aerogenes group which may be brought out by means of citric acid.

Bacterium coli was unable to make use of any of the acids with the possible exception of glutaric and here the results were questionable. Some of the cultures of soil origin readily utilized tricarballylic and aconitic acids though others did not possess this property. Glutaric acid was utilized sparingly if at all while adipic acid gave negative results.

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THE PRODUCTION OF CATALASE BY AN ANAEROBIC ORGANISM

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The production of catalase by bacteria has been well known since the discovery of this enzyme, and the early recognition of its almost universal occurrence in living cells and tissues. Beyerinck (1893), however, long ago showed that it is not produced in demonstrable quantities by the lactic acid producing group of bacteria, and Orla-Jensen (1919) and others have followed Beyerinck in giving considerable weight to this characteristic in the differentiation of the true lactic organisms. In fact, McLeod and Gordon (1923) have suggested a classification of bacteria based upon catalase production together with the ability or inability to form hydrogen peroxide.

Recently catalase formation has taken on an especial significance in studies of the obligatory anaerobic bacteria, owing to the non-production of this enzyme by the well known anaerobes and the probable physiologic importance of this fact. According to McLeod (1925), Löwenstein (1903) was the first to note the absence of catalase in an anaerobe. Callow (1923) found all of the anaerobic cultures which she tested to be devoid of catalase. These observations on the absence of catalase production among the obligate anaerobes have been confirmed and extended by McLeod and Gordon (1923) and by Avery and Morgan (1924). Hagan (1924) has added the significant finding that a strict anaerobe belonging to an entirely different group from the commonly studied sporulating anaerobes, *Actinomyces necrophorus*, also lacks the ability to produce catalase.

The plausible hypothesis has been advanced that, since hydrogen peroxide is apparently not formed in significant quan-

tities under anaerobic conditions, the reason the obligate anaerobes do not grow in the presence of free oxygen is because of their inability to produce catalase to effect the destruction of the peroxide formed. Many relevant data have been recorded in support of this view; forming evidence so strong, in fact, that this interpretation appears to be the most logical explanation of the anaerobic natures of at least some members of this class of organisms. Such a view would clarify such facts as the apparent aerobic growth of anaerobes in the presence of plant or animal tissues, and their growth in contact with air when associated with certain aerobes.

Lest a general theory of anaerobiosis be constructed upon this foundation, it is perhaps pertinent to call attention to an apparent exception to the general rule. The group of propionic acid producing bacteria, at least the strains with which the present writer is acquainted, should be classed as strict anaerobes but these organisms are especially vigorous producers of catalase. Attention has been called to this marked characteristic of the propionic bacteria in connection with discussions of their significance in the Swiss cheese industry (Sherman (1919, 1921)).

The note here recorded conflicts in no way with the excellent researches on the correlation between anaerobic growth and inability to form catalase, nor with the interpretation which other workers have put on these facts. It indicates, however, that all obligate anaerobes are not devoid of catalase producing capacity, and that an hypothesis which seeks to explain anaerobiosis on that basis is probably not universally applicable.

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STUDIES IN MICROBIC HEREDITY

VII. OBSERVATIONS ON THE GENETIC ORIGIN OF THE SEVERAL TYPES OF FUNGI FOUND IN THE LESIONS OF BLASTOMYCOSIS HOMINIS

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From the standpoint of infectious etiology there exists a curious paradox, in regard to the organisms of blastomycosis. A disease, which is both clinically and pathologically an entity, appears to be caused by a group of organisms comprising at least four distinct types which vary in their morphology from a budding yeast form, to a mould type having aerial hyphae with fruit organs, and reproducing by endosporulation.

Obviously it is difficult to reconcile this state of affairs with our views of the specific infective capacity of single types of bacteria for the clinical entities that they produce. In order to conform to this view Ricketts (1901) regarded the several types as more closely related than their diverse morphology might indicate. Yet there are others who take the position that these types are really distinct species and therefore find difficulty in explaining their relationship to the disease.

On the ground that these organisms comprise a more or less homogeneous group as Ricketts believed, there would seem to be no good reason why more than one type should not be isolated from the same case of the disease. It is entirely conceivable that one might find a different type of organism in the young lesion than in the old, inasmuch as it is in the latter that they have been under the perpetual assault of the immunologic forces of the host. Such a situation would amount to a dissociation of types within the host tissue, in which event one should be able to isolate them.

In point of fact paper VI of this series (1926) has shown that this possibility has been realized in the case studied. In order to prove, however, that the various types belong to a common stock it is necessary that a pure line culture of one type—be transformed into one of the other types. It is partly the purpose of this paper to report our experiments on the transformation of the Type II yeast into the mould-like Type III yeast.

VARIABILITY OF TYPE II

In the previous paper on these fungi (1926) I outlined the general conditions under which ascospore formation took place

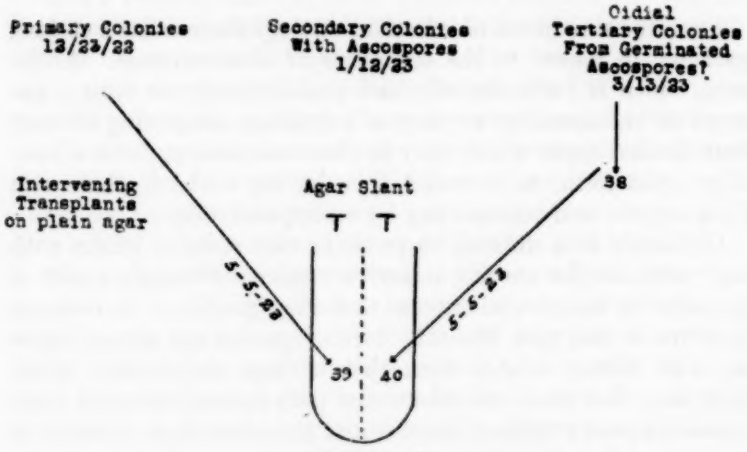

DIAGRAM 1.

and took occasion to refer to causative factors having to do with the strain itself which are not easily controlled.

With the aid of diagram 1 I shall now point my meaning by the elucidation of a most confusing paradox that we have observed on more than one occasion. This has to do with the fact that two strains of the same original pure line may later, when planted under identical conditions, yield life cycles of greatly varying degrees of complexity.

The Type II strain, transplant 33, was seeded on December 22, 1922, and developed on the dates indicated primary, secondary and tertiary colonies. Transplant 38 from the tertiary colonies preserved somewhat the larger oidial type of cell that characterized them. On May 5, 1923, as transplant 40 it was seeded on one side of the identical tube (*TT* in diagram) that received a seeding on its other side of the descendants of the primary colonies. The intervening transplants from the primary colonies were made on plain agar before the seeding of transplant 39 on May 5, 1923.

Transplant 40 grew rapidly and early formed a spreading mycelium. No. 39 grew slowly in discrete colonies with but slight tendency to form mycelium; yet there was morphologic identity in the cultures at this time. On June 15, 1923, no. 39 had developed a few insignificant secondary colonies in the upper or drying zone of the slant while no. 40 developed one large rough reddish secondary colony in the same zone.

On transplant this colony of no. 40 recapitulated the various steps in the anomalous method of reproduction shown in figures 16, 17, 18, 20 and 23 of paper VI of this series. On the contrary only the normal and oidial phases were seen in the secondary colonies of no. 39, and their transplants developed a cycle of relatively slight complexity. Yet the primary colonies of both nos. 39 and 40, as well as transplants from their secondary colonies, yielded the normal oval yeast phase of the organism, indicating the fundamental purity of both strains.

The essential difference between the two strains growing under exactly the same conditions involved the *range of their life cycles, and the origin of this difference appears to lie in the chromatic reconstructions of specialized cells* occurring in the secondary and tertiary colonies of transplant 33. The fact that these cells are of greatly increased viability is probably tributary to the major fact of their reorganization; indeed it seems possible that these chromatic changes require for their maturation a certain invulnerability of the cell.

Of great importance is the fact that the *descendants of these*

reorganized forms inherit some of the adaptability of which the forms themselves are the expression. This increased adaptability of their descendants is indicated by the readiness with which these phases of their life cycles are recapitulated. This expresses itself in secondary and at times in tertiary colonies. They signify that the pleomorphic cycle has been expanded to include special phases of development. Thus we have plausible explanation of the fact that two strains from the same original pure line, *planted under the same conditions*, may behave differently. The answer is simple: one strain has been rejuvenated through the characteristic reorganizations in the secondary colonies, but the other has not. One can only be assured of *keeping* hereditary lines pure if they are not permitted to pass through the sexual phase, or any other phase involving equivalent chromatic reorganization.

DIAGRAM 2.

Thus is explained in part *why* it may not be possible to formulate an environment that will permit the strains' expansion into all the *potential* phases of its cycle. If perchance the changes represented in diagram 1 occur in a strain without our observation, we are at a loss to explain the cyclic differences detailed above. The line is no longer pure even though the new biotype has characters that largely overlap those of the parent form. Had we been indifferent to the rôle of the secondary colonies we should have propagated the so-called normal phase only, and its lack of adaptability would have resulted in our loss of the strain. On the other hand, when the "normal phase" of any organism happens to be well adapted to ordinary media—as is indicated by its prolonged viability—the bacteriologist seldom sees the other phases, and when he does he calls them *involution* forms.

As further evidence for the microchemical reorganization that

such "involution" forms may undergo, are direct observations made on intravitally stained living forms.

The marked metachromasia shown by the nuclear material of these forms when stained in thionin arrested our attention. In many instances it stained a bright red, indicating a selective avidity for the polychrome component of the dye. This nuclear material is granular in form and may be located at either end of the cell or centrally (fig. 1 at *a*); some cells contained four of these nuclear collections. It is often sharply delineated from the rest of the cell and is surrounded by a clear space. The following changes were observed in cells staining diffusely blue.

The cell shown at *a* first became partially translucent as the result of concentration of the chromatin in bluish black granules (diagram 2 at *b*). These granules may at times migrate to the poles of the cell, or they may collect in its center. They were located in a large clear area and resembled somewhat a yeast nucleus (diagram 2 at *c*). The granules were actively motile and moved to all parts of the clear space.

In one of the larger cells containing four such collections two of them were seen to fuse much as drops of water run together (diagram 2 at *d*). Rather large bluish black granules broke up rapidly into a fine granular material, which in the twinkling of an eye became bright red in color. Within half an hour two rings of granules again appeared but the bright red color persisted (diagram 2 at *e*).

This changed chromatin at the end of the cells has been observed to initiate and actually to take part in the regular budding process of the cell (fig. 1 at *b*). Therefore it seems fair to conclude that this metachromatic reaction indicates a biochemical reconstruction on the part of the nuclear chromatin. It is probable, too, that it is of importance in the vital activities of the cell else it would not be concerned in reproduction. The rapidity with which this change from blue to bright red took place suggests physico-chemical alterations of a tauto-isomeric nature whose possible relation with chromatic reorganization I have detailed in a previous paper (Mellon, 1920).

THE BIOLOGIC ORIGIN OF THE MOULD TYPE NO. (III) FROM TYPE NO. II

We have attempted to indicate in our previous papers that with the bacteria the origin of new variants is associated with a type of chromatic reorganization similar to that shown in the secondary colonies of these fungi. Since the latter have yielded so many well recognized types of special growth forms it is not surprising that they have also proved the seat of origin for the mould-like stage of these fungi.

The poor viability of the normal forms of this strain has been an aid rather than a hindrance to the detection of the parent form of the new type. As nearly as can be pictured it is represented at *a* in figure 2. Normal sized forms are seen at *b*. The origin of this mould-like type has not been definitely traced to the zygospore—in fact we have seen no definite evidence of the latter process under the conditions of our observations.

The structures look like an oidium with a wall of great thickness. They recall the encysted oidium described by Guilliermond (1920). They closely resemble, too, certain chlamydospores, depicted in figure 25 of our previous paper (no. VI—1926) on these fungi. In any event they were the only forms in the culture capable of germination. This was proved not only by the fact that the normal forms looked dead and did not stain; it was shown also by the single cell technique, for regardless of how many normal cells were planted no growth occurred unless among them was one or more of the oidial-like structures just described.

The protocols detailing the origin of the variant are of special interest in connection with the expanded life cycle already described for transplant 33 (diagram 1). On January 24, 1923, a transplant was made from the ascospore containing secondary colonies of no. 33 to a partially dry agar slant to which 1 per cent maltose was added. The medium was a veal infusion, buffered to pH 6.0 with glycerophosphate and contained no pepton. The usual normal forms of the organism developed. The culture was sealed and kept at room temperature until September 10,

1923, when numerous fine black specks peppered the slant. In the absence of *sizable* secondary colonies these black specks, consisting of pigmented oidial forms, were not uncommon. Microscopically they appeared as the modified oidia referred to.

On transfer they rapidly grew into a spreading mycelium as seen in figure 3; they contrast sharply with Type II as seen in figure 4, in not yielding a creamy mass of buds starting from the center of the mycelial growth. From these mycelia spring long rows of oidial formation as seen in figure 5. Grossly, they appear as sharp tufts projecting from the surface of the culture.

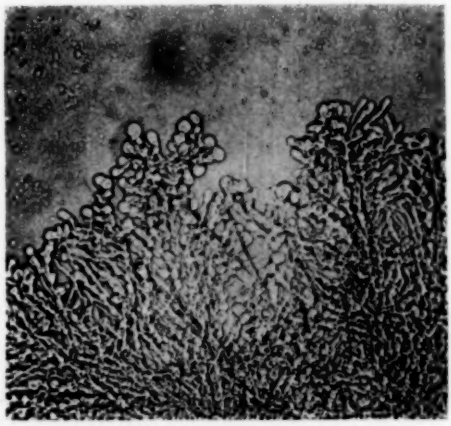

FIG. 5. THE VARIANT TYPE III SHOWING THE ORIGIN OF THE OIDIUM FROM THE MYCELIUM (FRESH UNSTAINED PREPARATION)
At this time "normal" germination was in abeyance. × 200

Definite aerial hyphae also form, but to date no fructification organs have developed from them.

For more than a year transplants of this culture reproduced only as the mycelial or the mycelial-oidial growth. Then buds began to originate from the mycelium identical morphologically with the normal forms of the maternal Type II. Secondary red colonies comprised of oidia indistinguishable from those of the black secondary colonies of Type II are not uncommon. When first dissociated the viability of Type III was fine, but

prolonged artificial cultivation has so decreased this that at present we are quite dependent on the red secondary colonies, unless we transplant at frequent intervals.

In the years that we have had this strain under close surveillance we have noted that this same variant has been reproduced six times. It has not always taken place on the same medium but the cultures from which it sprang have always been single cell ones. The change has been more or less spontaneous in that we did not start out to dissociate the variant. In fact many of these changes have come to notice by way of very close observation of what the organisms were doing *naturally*. Our attitude toward them has been not so much one of dictation as of "watchful waiting."

It is quite revealing what can be learned of microörganisms by merely transferring one's own cultures—not in the haphazard way of many modern technicians but with an eye always alert for the infinity of interest that living beings manifest. There can be no question that many beautiful anomalies are constantly discarded as contaminations by the unthinking technician; and to the extent that all of us cast aside from our daily routine the materials of perhaps valuable inquiry, we *are* technicians, regardless of our official status.

It was of interest that the antecedents of the Type III variant developed on a medium containing no pepton, but only germinated as the new form on a pepton-containing medium. Later the culture became adapted to the non-pepton media but without reverting to Type II. This illustrates a principle frequently repeated in our experience, and one that the mycologist understands better than the bacteriologist.

It can only mean that if we wish specialized stages to germinate we must frequently meet the organisms half way by providing an environment that will be compatible with the reorganizations which these special stages represent. On the other hand it must be appreciated that the reorganizations must be given a chance to ripen or mature, else they will not germinate on *any* medium. Only recently my Type III mould-like blastomyces failed to germinate, but a microscopical examination suggested

that immature oidia were developing from the mycelia. In about a month these blossomed forth as beautiful red secondary colonies and from them the original form of the Type III was then easily recovered. How easy it would have been to have discarded the culture as dead!

REACTIONS OF TYPE II AND TYPE III WITH SUGARS

Technique

Sugar free broth was prepared after Theobald Smith's method. To tubes containing 9 cc. of broth was added 1 cc. of 10 per cent

TABLE 1

TIME	GLUCOSE		LEVULOSE		GLYCEROL		LACTOSE		MALTOSE		MANNITOL		SALICIN		CONTROLS	
	Type II	Type III	Type II	Type III	Type II	Type III	Type II	Type III	Type II	Type III	Type II	Type III	Type II	Type III	Type II	Type III
days																
7	6.1	6.3	6.0	6.3	6.2	6.4	6.0	6.3	6.0	6.2	6.3	6.2	6.0	6.3	6.7	
	6.0	6.2	6.0	6.3	6.2	6.5	6.2	6.4	*6.0	6.2	6.3	6.3	6.2	6.4	6.8	
14	6.1	6.7	6.1	6.3	6.5	7.1	6.7	7.0	6.0	6.2	6.6	6.9	6.4	6.2	6.8	
	6.2	6.8	6.2	6.4	6.5	7.1	6.7	7.1	6.1	6.1	6.5	7.0	6.4	6.2	6.8	

* Methyl red used as control indicator.

solution of the various sugar solutions. They were grown for seven days at room temperature and titrated, and a duplicate set was titrated after fourteen days. The colorimetric method was employed. The results are in terms of pH. The indicators chiefly used were bromo-thymol blue and brom-cresol purple. Duplicate readings were made.

It is clear from table 1 that of the seven fermentable substances tested all were fermented with moderate acid production but no gas (one week). With lactose, Type II showed reversal of reaction at the end of two weeks, which was also partial for glycerol. Complete reversal or even alkali formation occurred with Type III in glucose, glycerol, lactose and mannitol. So although there is great similarity in the fermentative capacity of the

strains there is apparently some qualitative difference in the end products formed. The gross differences of the cultures are, to an extent, belied by the similarity of these tests which confirm their genetic lineage.

COMPLEMENT FIXATION RESULTS

The strains isolated from the patient were tested out serologically with his serum as well as with the sera of rabbits which had been inoculated with them. The agglutination results could not be regarded as satisfactory so fixation antigens were made of Types I and II. After a good growth had been obtained the organism was washed in salt solution and 0.1 per cent formol added. It was controlled for growth after four or five days and if sterile tested for its anti-complementary powers. If suitable, the antigenic dose of the antigen was determined by choosing one-fourth of the anti-complementary dose with normal serum. The antigenic unit in each case resulted in 0.1 cc.

When run against the patient's serum in 0.3 cc. a 4+ reaction with Type I antigen was obtained after thirty minutes incubation. A 3+ reaction was observed with Type II. After one hour, however, the reaction was only between 1+ and 2+. This delayed type of reaction and its probable significance I have already described in a previous communication (1921). Animals immunized against Type I organism produced a serum which fixed complement in 4+ in $\frac{1}{10,000}$ cc. in one rabbit and $\frac{1}{10,000}$ cc. in another. A strain of *Endomyces albicans* (the thrush fungus) which had been isolated from an infected finger by Tanner gave a 4+ reaction in 0.1 cc. of the rabbit's sera but was negative in 0.01 cc. It is of interest that the heterologous Type II antigens showed greater fixing power for the serum of animals with Type I than did the Type I antigen. This occurrence is frequently paralleled, however, in agglutination work with bacteria.

DISCUSSION

As the result of our isolation of Types I and II blastomyces from a single case, coupled with the experimental transforma-

tion of Type II to Type III, Ricketts' original view of the close genetic relations of these types is confirmed. The law of Koch which predicates a morphologic type specificity for strains of organisms cannot be regarded as absolute. These morphologic types scarcely correspond to the *species* of the higher forms. They represent instead stages which have been stabilized or dissociated from the pleomorphic cycle of possibly a single entity.

Because the situation is strictly comparable to what our previous studies (1925–1926) have indicated for the bacteria the results have a peculiar interest. The parallelism with study no. III, which shows the true pleomorphism of *B. alkaligenes*, is especially noteworthy here because in its stabilized coccus, bacillus and filamentous branching types we have much the same complex as is presented by the morphology of the three types of blastomyces (endomyces). With the bacteria the complex is of a lower order of magnitude in the same sense that the solar system of the atom represents a lower order of magnitude than the solar system of the universe. In each instance the fundamental principle of arrangement is preserved.

There is another aspect of the situation that merits philosophic treatment since it has a really practical value in genetic work. I refer now to the difficulty of drawing a hard and fast line between environmental and intrinsic factors as they may determine variation within pure lines of organisms. Since it is practically impossibly to conceive of the existence of a living organism apart from an environment, I do not see how any one can limit variability changes *with certainty* to strictly intrinsic factors. Strictly speaking we cannot control environment since we are not even aware of all of its factors.

In our experience alterations in the hereditary mechanism occur in special stages of the organism, viz., in zygospores or their reorganization equivalents. The unfolding of these stages is conditioned by the environment at the time, *or by a previous environment*. So the behavior of an organism (in these respects) under a given environment could not be predicted short of an intimate knowedge of its full genetic history.

Eberson (1918) took the position that "different types, to be genuine mutants, must have developed when conditions remained constant." Aside from the great difficulty of knowing when environmental factors actually remain constant, this dictum is not consonant with the facts, as far as our experiments go. Neither does it conform with experiments of recent years in the field of experimental biology as I have previously pointed out (1920). There can be little question that a changing environment is of much significance in variational change, particularly when it is brought to bear on the germ plasm.

In confirmation of the isolation of more than one type (stabilized stage) of fungus from different lesions of the same patient we have the work of Rappaport and Kaplan reported at the (December, 1925) Meeting of the American Society of Bacteriologists. From a case of meningitis diagnosed antemortem by positive blood and spinal fluid cultures they obtained two types that parallel our Types I and II. They obtained them from different organs and have shown antigenic relation between them.

CONCLUSIONS

1. Following isolation of Types I and II yeasts from a single case of blastomycosis, the mould-like Type III has been derived genetically from pure line cultures of Type II.

2. The botanical mechanism for this transformation has been associated with specialized cells in the pleomorphic cycle of the organism. They resemble somewhat the chlamydospores.

3. The variation mechanism in general is the same as with the bacteria, although with the yeasts no definite evidence of true isoganic conjugation was observed in this instance.

4. The origin of very closely related biotypes also seems to be identified with the chromatic reorganizations of the pleomorphic cycle.

5. When conjugation or similar change occurs there can be no guarantee that the culture is *then* a pure line one.

6. The original view of Ricketts that the three distinct types really comprise a rather homogeneous group is sustained by actual experiment.

7. The isolation of Types I and II from lesions of different age makes it highly probable that variations occur in the host similar to those induced in vitro.

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EXPLANATION OF PLATE I

FIG. 1. METACHROMATIC REORGANIZATIONS OF NUCLEAR CHROMATIN IN GIANT YEAST CELLS AT *a*, WHICH AT *b* PARTICIPATES IN THE GERMINATION OF A NEW CELL

Intravitally stained in carbol-thionin. × 800

FIG. 2. REORGANIZATION FORMS OF SPECIAL RESISTANCE GROWING ON 2 PER CENT NaCl AGAR

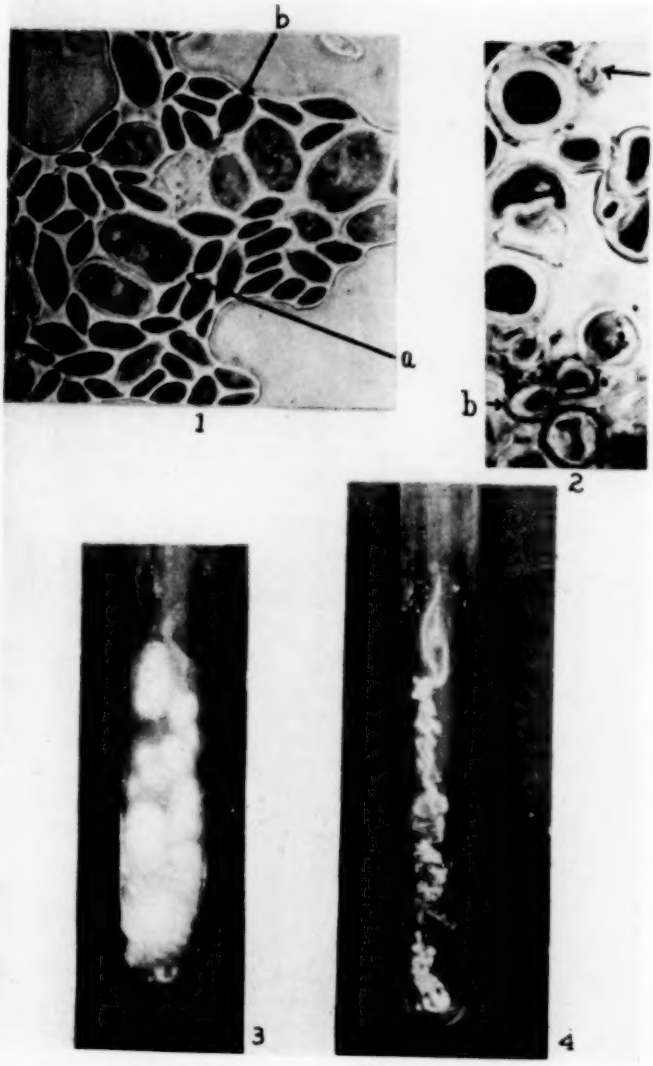
At *a* the parent cells for transition of Type II to the mould-like Type III. At *b* the poorly staining non-viable "normal forms." Carbol-thionin. × 800.

FIG. 3. THE MOULD-LIKE TYPE III DERIVED GENETICALLY FROM THE TYPE II YEAST

Note dominance of mycelial phase

FIG. 4. TYPE II

Note dominance of budding phase, which has overshadowed the scant mycelium which develops early.



(Mellon: Studies in microbic heredity)